Toxicology and Applied Pharmacology 161, 23–33 (1999)
Article ID taap.1999.8788, available online at http://www.idealibrary.com on IDEAL®

4-Hydroxynonenal and Malondialdehyde Hepatic Protein Adducts in Rats Treated with Carbon Tetrachloride: Immunochemical Detection and Lobular Localization

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Received May 21, 1999; accepted August 25, 1999

4-Hydroxynonenal and Malondialdehyde Hepatic Protein Adducts in Rats Treated with Carbon Tetrachloride: Immunochemical Detection and Lobular Localization. Hartley, D. P., Kolaja, K. L., Reichard, J., and Petersen, D. R. (1999). *Toxicol. Appl. Pharmacol.* 161, 23–33.

The metabolism of CCl_4 initiates the peroxidation of polyunsaturated fatty acids producing α,β-unsaturated aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). The facile reactivity of these electrophilic aldehydic products suggests they play a role in the toxicity of compounds like CCl_4. To determine the rate at which CCl_4-initiated lipid peroxidation results in the formation of 4-HNE and/or MDA hepatic protein adducts, rats were given an intragastric dose of CCl_4 (1.0 ml/kg) and euthanized 0–72 h after administration. Rabbit polyclonal antisera directed toward 4-HNE- or MDA-protein epitopes were employed in immuno-histochemical and immuno-precipitation/Western analyses to detect 4-HNE and MDA-protein adducts in paraffin-embedded liver sections and liver homogenates. As early as 6 h post CCl_4 exposure, 4-HNE and MDA adducts were detected immuno-histochemically in hepatocytes localized to zone 2 of the hepatic acinus. Liver injury was progressive to 24 h as lipid peroxidation and hepatocellular necrosis increased. The hallmark of CCl_4 hepatotoxicity, zone 3 necrosis, was observed 24 h after CCl_4 administration and immuno-positive hepatocytes were observed in zone 2 as well as zone 3. Immuno-positive cells were no longer visible by 36 to 72 h post CCl_4 administration. From 6 to 48 h after CCl_4 administration, at least four adducted proteins were immuno-precipitated from liver homogenates with the anti-MDA or anti-4HNE serum, which corresponded to molecular weights of 80, 150, 205, and greater than 205 kDa. These results demonstrate that 4-HNE and MDA alkylate specific hepatic proteins in a time-dependent manner, which appears to be associated with hepatocellular injury following CCl_4 exposure.

Key Words: carbon tetrachloride; lipid peroxidation; hepatotoxicity; malondialdehyde-4-hydroxynonenal-protein adducts; oxidative stress.

Carbon tetrachloride (CCl_4) is a predictable and prototypic zone 3 hepatotoxicant. Within hours after the administration of CCl_4, experimental animals display hepatic steatosis and centrilobular necrosis. Central to the plethora of mechanisms implicated in the early phases of this chemical-induced liver injury is the reductive metabolism of CCl_4 by cytochrome P-4502E1, which generates the chemically reactive, carbon-centered trichloromethyl radical, $Cl_3C^{\cdot}$ (Williams and Burk, 1990). The interaction of this radical with hepatic lipids (Trudell *et al.*, 1982) and proteins (Recknagel, 1983) has been confirmed. However, the specific role of protein or lipid adduction in CCl_4- mediated liver injury has not been delineated and only recently has it been proposed that the mitochondria is a sensitive target of CCl_4-induced hepatocellular injury (Hernandez-Munoz *et al.*, 1992).

The potential for chemicals to modify cellular protein through covalent modification is an established mechanism of chemical toxicity. As noted in a recent review (Cohen *et al.*, 1997) alkylation of hepatic proteins is a well-documented event following acetaminophen overdose and is also reported to occur in response to the hepatotoxicity associated with halothane (Sato *et al.*, 1985), diclofenac (Hargus *et al.*, 1994), trichloroethylene (Halmes *et al.*, 1996), and the antibiotic sulfamethoxazole (Cribb *et al.*, 1996).The alkylation of hepatic proteins by acetaldehyde (Isreal *et al.*, 1986) or the hydroxyethyl radical metabolite of ethanol (Clot *et al.*, 1997) are also proposed to be important mechanisms in alcohol-mediated liver injury. In these instances the metabolic bioactivation of each parent compound results in drug-derived protein-metabolite conjugates that can be detected immuno-chemically with antibodies against these specific protein-chemical haptens.

Alternatively, chemicals such as iron, ethanol, and CCl_4 initiate production of electrophilic aldehydes through oxidative degradation of biological membranes. These chemically medi-

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[2] Supported by NIH/AA05370 to D.P. Hartley and AA09300 to D.R. Petersen.

0041-008X/99 $30.00

ated events result in the α,β-unsaturated aldehydic products of lipid peroxidation, 4-HNE and MDA, both of which can react with cellular nucleophiles and possibly elicit toxicity. Iron, alone or in combination with ethanol, administered to rats results in the appearance of MDA and/or 4-HNE-adducted proteins in liver sections (Tsukamoto *et al.*, 1995). Similarly, both MDA and/or 4-HNE adducted proteins were observed in liver biopsy specimens obtained from humans with chronic liver disease attributable to chronic ethanol consumption, iron storage diseases (Paradis *et al.*, 1997a), and chronic hepatitis C (Paradis *et al.*, 1997b).

The potential for CCl_4 to initiate lipid peroxidation has been repeatedly documented in cellular systems (Danni *et al.*, 1991) and a variety of animal models (Williams and Burk, 1990). However, there are a limited number of published reports describing the detection of adducted hepatic proteins following CCl_4 treatment. Immuno-histochemical procedures using antibodies directed against MDA-adducted low-density lipoprotein (LDL) have been used to detect hepatic MDA-adducted proteins in liver sections of rats treated with CCl_4 (Bedossa *et al.*, 1994). These investigators noted that immuno-positive hepatocytes increased in intensity for 48 h following CCl_4 administration and were localized primarily to zone 2, and progressed to zone 3. Also, the density of the immuno-positive cells decreased over a 7-day postexposure period. Collectively, the data presented by these investigators are noteworthy in that they were the first to describe an association between the degree of lipid peroxidation, MDA-adducted immuno-positive hepatocyte staining, and hepatocellular damage. Although the report of Bedossa *et al.* (1994) qualitatively described the appearance of MDA-derived adducts in liver slices from CCl_4-intoxicated rats, the data described in this communication are the first to characterize proteins alkylated by MDA or 4-HNE in liver sections and homogenates prepared from rats treated with CCl_4.

To further understand the toxicological significance of aldehyde generation and the subsequent alkylation of proteins, it is important to detect and eventually identify proteins that are targets for adduct formation by lipid aldehydes. The studies described herein establish the temporal relationship between CCl_4-initiated lipid peroxidation, hepatocellular damage, and the formation of 4-HNE- and MDA-hepatic protein adducts. Novel data are presented using immuno-histochemical detection of aldehyde-adducted proteins in liver sections as well as immuno-precipitation and immuno-blotting procedures to detect and initially characterize 4-HNE and MDA-adducted proteins in liver homogenates prepared from rats treated with CCl_4.

METHODS

Chemicals, biochemicals, reagents, and solutions. All solutions were prepared using deionized and distilled water. The following chemicals and biochemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO), including: butylatedhydroxytoluene (BHT), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), thiobarbituric acid (TBA), sodium pentobarbital, light mineral oil (paraffin oil), protein A-sepharose CL-4B, benzamidine, phenylmethylsulfonylfluoride (PMSF), ethanolamine, 0.1% poly L-lysine solution, Mayer's hematoxylin, and all chemicals for electrophoresis. Diaminobenzidine (DAB) was purchased from Pierce (Rockford, IL). Goat anti-rabbit IgG conjugated with horseradish peroxidase and streptavidin conjugated with horseradish peroxidase were purchased from Gibco-BRL (Life Technologies, Inc., Gaithersburg, MD). Carbon tetrachloride was from Aldrich Chemical Co. (Milwaukee, WI). Aprotinin, pepstatin, leupeptin, antipain, RNase, and DNase were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Absolute ethanol was from Aaper Alcohol and Chemical Co. (Shelbyville, KY).

Animals. Male HAS (highly alcohol sensitive) rats (200–300 g) were obtained from the University of Colorado Alcohol Research Center. As described elsewhere (Draski *et al.*, 1992), these animals have been selected to genotypically express specific behavioral responses after acute ethanol administration that are independent of the enzymatic pathways involved in ethanol and acetaldehyde metabolism. Thus, the phenotypic responses of these rats to hepatotoxins such as CCl_4 are predictably similar to other genetic stocks of rats. All animals received humane care. The animal experimental protocols described were reviewed, consistent with National Institutes of Health guidelines, and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Health Sciences Center.

Prior to treatment, each animal was fasted overnight and animals received an intragastric dose (1.0 ml/kg) of CCl_4 in a volume of mineral oil equivalent to 10 ml/kg (Hjelle *et al.*, 1983). Control animals received mineral oil intragastrically. At predetermined time points after administration of CCl_4 or mineral oil, each animal was anesthetized with sodium pentobarbital (65 mg/kg ip).

Hepatotoxicity was assessed by serum alanine aminotransaminase (ALT) activity. Prior to extraction of the liver, blood samples (5.0 ml) were obtained from each rat by cannulating the descending aorta. Samples were stored at 4°C for at least 24 h. Whole serum was collected from the samples and used for determination of ALT activity. This assay was performed spectrophotometrically as per the directions specified by the manufacturer (Sigma, Procedure 59-UV).

Tissue isolation and subcellular fractionation. Livers were excised from anesthetized rats and immediately immersed in a solution of precooled (4°C) 0.25 M sucrose/0.1 mM BHT. One gram of liver was placed in 9 volumes of cold (4°C) sucrose/BHT (9:1; buffer:liver; v/w) containing protease inhibitors and homogenized with six passes of a teflon pestle in a 50-ml Potter-Elvehjem glass homogenizing tube. A small aliquot of the 10% homogenate was analyzed for protein content by the Biuret assay (Gornall *et al.*, 1949). The remaining homogenate was immediately frozen in 50-ml Falcon tubes at −80°C.

Assessment of thiobarbituric acid reactive substances (TBARS) as an index of hepatic lipid peroxidation. TBARS were measured in 10% liver homogenates (Buege and Aust, 1978). Briefly, livers from CCl_4-treated rats were homogenized (1:9 w/v) in 0.25 M sucrose/BHT as described above and an aliquot of each homogenate (1.0 ml) was mixed with an equivalent volume of 30% TCA. Each sample was derivatized with 50 mM TBA (1.0 ml). Samples were mixed with 50 mM TBA and heated at 100°C for 15 min. Those samples from sucrose-suspended homogenates were also heated, but at 80°C for 15 min. This lower temperature results in decreased sucrose interference in the TBA assay (Huber *et al.*, 1975). After heating, the samples were removed from the water bath and centrifuged at 2000 rpm for 10 min to pellet precipitated protein. Derivatization blanks (without homogenate) containing only TBA and KPO_4 buffer or TBA and sucrose were processed simultaneously to the experimental samples to correct for buffer- and sucrose-derived TBA absorbance. The levels of TBARS were quantified spectrophotometrically at 536 nm using an extinction coefficient for the TBA-derivative of MDA of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

Synthesis of haptens, hapten-carrier protein conjugates, and antibody production. Polyclonal antibodies directed against 4-HNE-sulfhydryl adducts were prepared by immunizing rabbits with a protein carrier conjugate of 4-HNE and *N*-succinimidyl-*S*-acetylthioacetate (SATA)-activated KLH (Hartley *et al.*, 1997). Likewise, lysine-rich KLH adducted with MDA was used to obtain polyclonal antibodies against MDA-amine epitopes. The detailed chemical synthesis of these antigens and characterization of the polyclonal antibodies is described in detail elsewhere (Hartley *et al.*, 1997). These polyclonal antibodies have been successfully used in our previous studies to characterize aldehyde adducted proteins in isolated hepatocytes exposed to CCl_4 (Hartley *et al.*, 1997) and, as a result, expedited adaptation and validation for the studies described herein.

Immuno-histochemical detection of 4-HNE- and MDA-protein adducts in tissue sections. Small sections (~0.5 cm^3) of freshly extracted rat liver were dissected from the liver and immediately fixed in 10% neutral-buffered formalin (270 ml 37% formalin, 4.0 g monobasic $NaPO_4$, 6.5 g dibasic $NaPO_4$ in 900 ml water). Preserved tissue was washed, dehydrated with ethanol, and embedded in paraffin at Colorado Histo-Prep (Fort Collins, CO). Tissue sections (4 μm) were placed on poly L-lysine-coated microscope slides. Prior to immuno-histochemical staining, each section was deparaffinized and rehydrated in a series of solvents (xylene, absolute ethanol, 95% ethanol, and water; 2× each). Endogenous peroxidase activity was blocked by exposure to H_2O_2 (3% in distilled/deionized water; v/v) for 15 min. Nonspecific antibody interactions were blocked with normal goat serum (1:75 in PBS, pH 7.4). All 4-HNE- and MDA-protein adducts were detected with antisera directed to either adduct (1:500 in PBS, pH 7.4). Immunopositive interactions were indirectly detected with goat anti-rabbit IgG conjugated with biotin (1:200 in PBS, pH 7.4; Gibco-BRL) followed by streptavidin conjugated with horseradish peroxidase (1:2000 in PBS, pH 7.4; Gibco-BRL). Visualization of these interactions was with diaminobenzidine (1 mg/ml in PBS, pH 7.6; Pierce)/H_2O_2 (1 μl/ml). Alternatively, the same results could be generated using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500 in PBS, pH 7.4; Gibco-BRL) followed by diaminobenzidine detection. Preimmune sera (1:500) or secondary antisera (1:500) were substituted for epitope specific antisera in control experiments.

Quantification of histopathology and immuno-histochemistry. Necrotic and immuno-positive cells were quantified by light microscopy (400×) in histochemical and immuno-histochemical tissue sections, respectively. In all evaluations, at least 1000 cells were counted per section and necrotic or immuno-positive cells were noted. These counts were performed only on sections containing lobular regions with intact, clearly identifiable periportal and central lobular regions. Zonation of necrotic or immuno-stained hepatocytes was based on the assumption that zone 3 hepatocytes occupied one third of the cell mass surrounding the central vein while those hepatocytes representing one third of the cell mass surrounding the portal vein were classified as zone 1 hepatocytes. Those hepatocytes intermediate to zone 1 and zone 3 were designated zone 2. An outside investigator who was unaware of the treatment of the tissue sections scored all slides.

Immuno-precipitation of proteins alkylated by 4-HNE or MDA. Immuno-precipitation (IP) of hepatic proteins adducted by 4-HNE or MDA was performed by mixing the liver homogenates (volumes equivalent to 2 mg) with an equivalent volume of immuno-precipitation buffer (Hartley *et al.*, 1997) and incubating this mixture with the antiserum (1 μl/100 μl of mixture). In a separate tube, protein A-sepharose CL-4B agarose beads (Sigma) were hydrated in distilled water and resuspended in 1% BSA IP buffer. Immunoprecipitations were incubated overnight at 4°C and, the following day, 50 μl of a 6% (w/v) suspension of protein A–sepharose CL-4B beads in IP buffer was added and agitated for an hour at room temperature. Following immunoprecipitation with protein A–sepharose, the beads were washed three times with IP buffer and a single final wash with 10 mM Tricine. Electrophoresis sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue) was added to each tube and samples were incubated at 100°C for 10 min and then applied to SDS–PAGE (Hartley *et al.*, 1997).

Western-blot analysis of 4-HNE- and MDA-modified proteins. Proteins separated by SDS–PAGE were transferred to nitrocellulose in 192 mM glycine, 25 mM Tris–HCl, 1.3 mM SDS, pH 8.2, over 2 h at 0.7 A. Standard Western blotting procedures were used as described elsewhere (Hartley *et al.*, 1997). All antibodies and reagents were diluted in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Positive interactions were visualized with the enhanced chemiluminescence substrate for horseradish peroxidase (Amersham-ECLR).

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) with time and treatment variables to detect differences in the various biochemical parameters over the time course of pro-oxidant exposure. Post-hoc comparisons of mean values for determination of significant differences (significance level of $p < 0.05$ or greater) was by the Tukey *b* test. All data are presented as means ±1 SEM. These analyses were conducted using the Crunch Version 4, Statistical Package (Crunch Software Corp., Oakland, CA).

RESULTS

Histologic and biochemical indices of liver injury. In our initial studies, corn oil was evaluated as the vehicle for CCl_4 administration. However we observed that, when administered alone, corn oil resulted in a mild but detectable liver injury. Therefore, mineral oil was selected as the alternative vehicle for CCl_4 administration. Based on hematoxylin-and-eosin-stained tissue sections, mineral oil administration caused a subtle but detectable centrilobular steatosis. In contrast, CCl_4 administered in mineral oil elicited extensive changes in liver morphology, including frank steatosis, inflammation, and necrosis. These predictable histopathological changes during the progression of CCl_4-initiated liver injury are documented in the photomicrographs presented in Figs. 1A–1D. Six hours after CCl_4 exposure, the livers appear quite normal. However, within 12 h, a significant number of ballooned hepatocytes are evident, as are inflammatory cells. Hepatic injury was progressive and from 18 to 48 h massive centrilobular (zone 3) steatosis, inflammation, and necrosis are observable (Figs. 1C and 1D). As illustrated in Fig. 2A, mineral oil caused a transient but significant elevation in serum ALT in control animals. In contrast, hepatic damage was significant in animals as early as 6.0 h after CCl_4 exposure, where CCl_4 caused a 5-fold greater increase in ALT compared to mineral oil. In addition, CCl_4 elicited progressive liver injury as indicated by increases in serum ALT levels, where, 36 h after exposure to CCl_4, serum ALT activity increased 32-fold above ALT values for mineral oil controls at the same time point. If rats were allowed to recover for longer periods of time after CCl_4 exposure, serum ALT levels decreased but were still substantially elevated 48–72 h after CCl_4 administration.

The photomicrographs presented in Fig. 1, in conjunction with the elevations of ALT depicted in Fig. 2A, document the general time-course and degree of CCl_4-induced liver injury. However, the quantitative data presented in Fig. 2B are definitive with respect to the time-course of cellular necrosis specific to periportal (zone 1), midzonal (zone 2), and centrilobular (zone 3) regions of the liver lobule. Following treatment with CCl_4, the number of necrotic cells in zone 1 increased

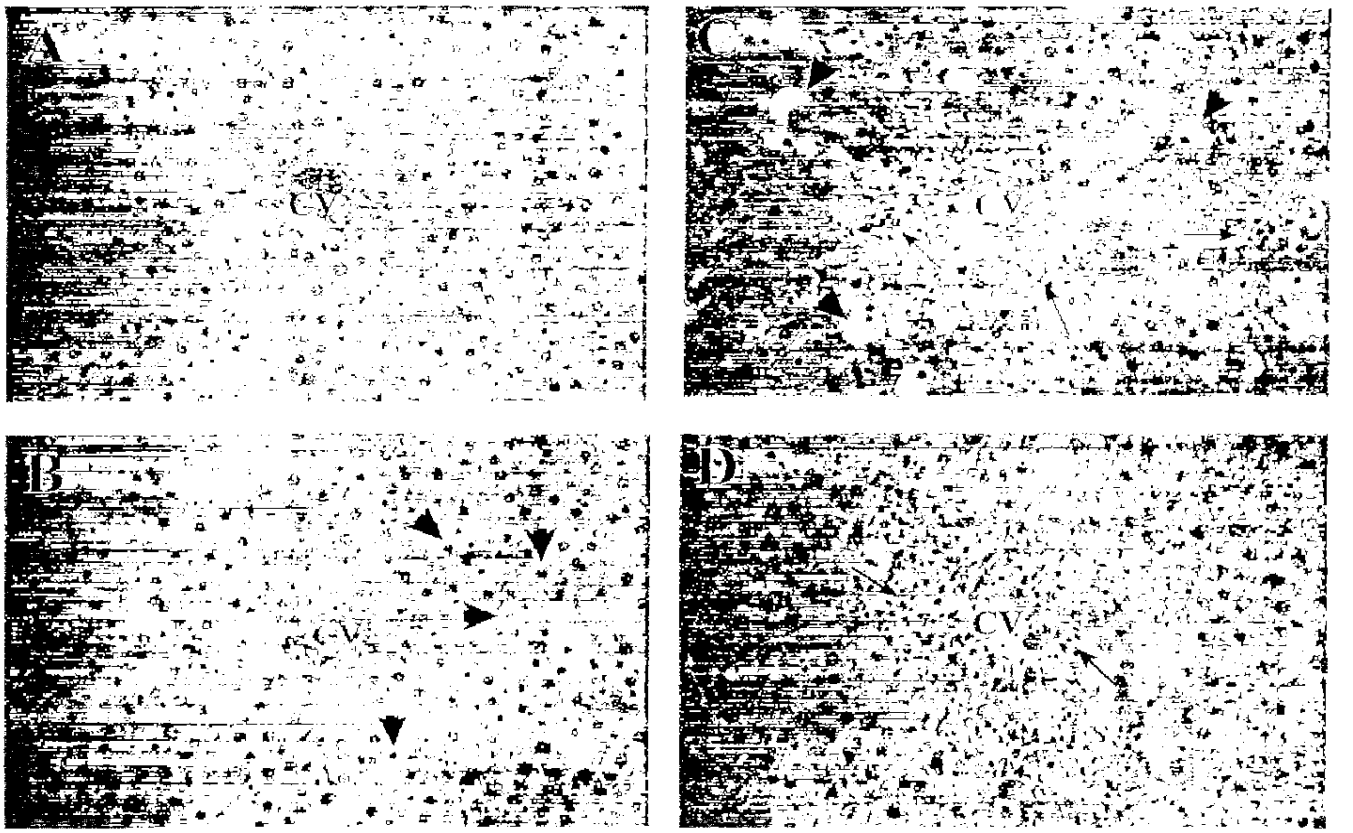


FIG. 1. Histopathological time course for the progression of CCl_4-initiated liver injury. (A–D) Representative hematoxylin-and-eosin-stained tissue sections obtained from animals at 6.0, 12, 18, and 48 h, respectively, after exposure to CCl_4. Photomicrographs (magnification 100×). A central vein (CV) is present in the center of each photomicrograph. Heavy dark arrows in B and C indicate ballooned hepatocytes. The smaller dark arrows in C point to foci of inflammatory cells. In D, light arrows designate zone 3 localization of necrotic cells. Note also in D the presence of numerous inflammatory cells in the centrilobular area.

slightly above baseline only at the 12-h time point. By 12 h following CCl_4 administration, the most apparent cellular necrosis observed was midzonal (zone 2), which was 2.5-fold greater than that observed in zone 3. From 12 through 48 h following CCl_4 administration, however, the number of necrotic cells present in the centrilobular region (zone 3) exceeded those present in zone 2 by approximately 2-fold. The predominance of necrotic cells present in zone 3 at the 36 and 48 h is certainly consistent with the classification of CCl_4 as a zone 3-selective hepatotoxicant.

TBARS were measured as a biochemical index of prooxidant-initiated lipid peroxidation (Fig. 3). Corresponding to indices of hepatotoxicity and changes in liver morphology, CCl_4-treatment in rats resulted in elevated levels of TBARS. The TBARS values in liver homogenates from rats treated with CCl_4 were maximal from 18 to 36 h, during which time they were elevated approximately 2.5-fold above those observed in liver homogenates prepared from rats administered mineral oil alone.

Immunohistochemical and lobular localization of MDA- or 4-HNE-adducted proteins. Lipid-derived aldehydes were detected as aldehyde-protein adducts in rat liver tissue sections after CCl_4 exposure using antisera developed against 4-HNE-sulfyhydryl- or MDA-amine-modified hepatic proteins. In rats treated with only mineral oil, a few hepatocytes were observed to contain 4HNE- or MDA-hepatic protein adducts in centrilobular regions of rat liver 12 to 24 h after exposure to mineral oil (data not shown). These adducts were most commonly colocalized with accumulated lipid droplets distributed in zones 2 and 3. Within 48 h, these adducts were not detectable, even though a very mild mineral oil-induced, centrilobular steatosis was apparent.

In rats administered CCl_4 intragastically, aldehyde-protein adducts were detected as early as 6 h after CCl_4 exposure (Figs. 4A and 4E). As is apparent from Fig. 4, midzonal (zone 2) hepatocytes displayed intense immuno-positive staining for both MDA-amine (Figs. 4A–4D) and 4-HNE-sulfhydryl adducts (Figs. 4E–4H) and the density of immuno-positive cells

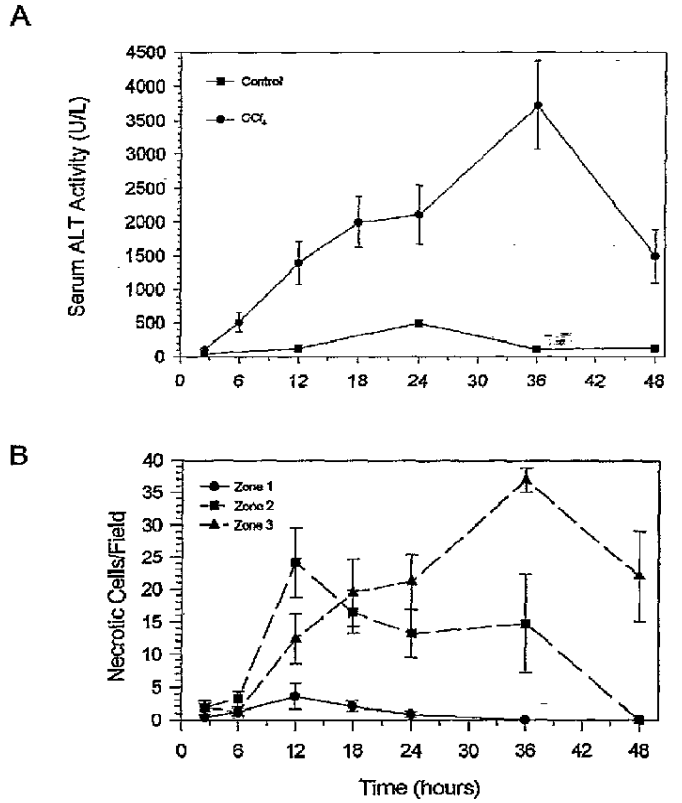


FIG. 2. Serum alanine aminotransferase activity and histopathologic quantitation of hepatic injury following acute CCl_4 administration to rats. (A) Profile of serum ALT index of hepatic injury that occurred 2.5 to 48 h after rats were administered mineral oil (solid squares) or CCl_4 in mineral (solid circles). (B) Summary of results obtained from liver sections of CCl_4-treated rats in which necrotic hepatocytes were quantified in each zone of the liver acinus. The data in A and B are presented as means ±1 SEM for three to seven animals.

increased with time up to 36 h post-CCl_4 administration. It is also evident that aldehyde-protein adducts appear in the lobule in a gradient fashion; formation occurs initially in the midzonal region (zone 2) with detectable but minor staining patterns observed in the centrilobular region (zone 3) at later times. As shown, both MDA- and 4-HNE-protein adducts appear to have similar distributions and, based on the time course evaluated here, have similar half-lives in the liver. When the animals are allowed to recover for 36 to 48 h after CCl_4 intoxication, the presence of adducted proteins in hepatocytes is much less extensive and is isolated to cells with altered cellular morphology.

Quantification of the immuno-histochemistry presented in Figs. 4A–4F was performed to establish the distribution of immuno-positive cells in zones 1–3 of the liver lobule and is presented in Figs. 5A and 5B. These data document an 8- to 10-fold abundance of MDA or 4-HNE immuno-positive cells in zone 2, 12 h after CCl_4 administration. These data demonstrate disappearance of MDA or 4-HNE immuno-positive cells by 24 h and also confirm a near absence of adducts in zone 3 during the 48 h after CCl_4 administration. Liver sections were also immuno-stained with preimmune serum and immuno-positive interactions were quantified (Fig. 5C). It is apparent that detection of immuno-positive cells is specific for MDA or 4-HNE epitopes and is independent of nonspecific interactions with rabbit serum.

Immunoblot detection of MDA- or 4-HNE-adducted proteins in liver homogenates. To detect specific target proteins for MDA or 4-HNE alkylation during CCl_4 initiated liver injury, liver homogenates prepared from rats administered mineral oil or CCl_4 in mineral oil were screened in immuno-precipitation–immuno-blot analyses (Figs. 6 and 7). Interestingly, mineral oil administration alone resulted in the formation of detectable and specific aldehyde protein adducts. With 4-HNE-sulfhydryl (Fig. 6) or antisera MDA-amine (Fig. 7), immuno-positive proteins of 80, 150, and 205 kDa or greater were apparent in control animals from 12 to 48 h. The basal immuno-reactivity of these proteins changed over the time course studied; however, the profile of adduct formation in samples from control animals was different and the intensity of these immuno-positive proteins was decreased compared to samples from CCl_4-treated rats. Formation of MDA and 4-HNE adducts may be attributed to the polyunsaturated fat content of mineral oil, which would provide ample substrate for autooxidation and thereby yielding mineral oil-derived aldehydic products of lipid peroxidation.

The data presented in Figs. 6 and 7 demonstrate that CCl_4 administration to rats results in the production of lipid aldehydes (i.e., 4-HNE and MDA) that alkylate specific hepatic proteins to a greater extent than in control samples. Immuno-

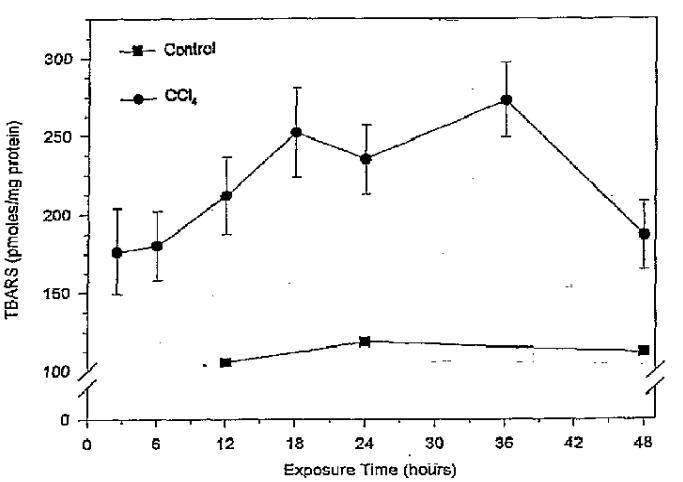
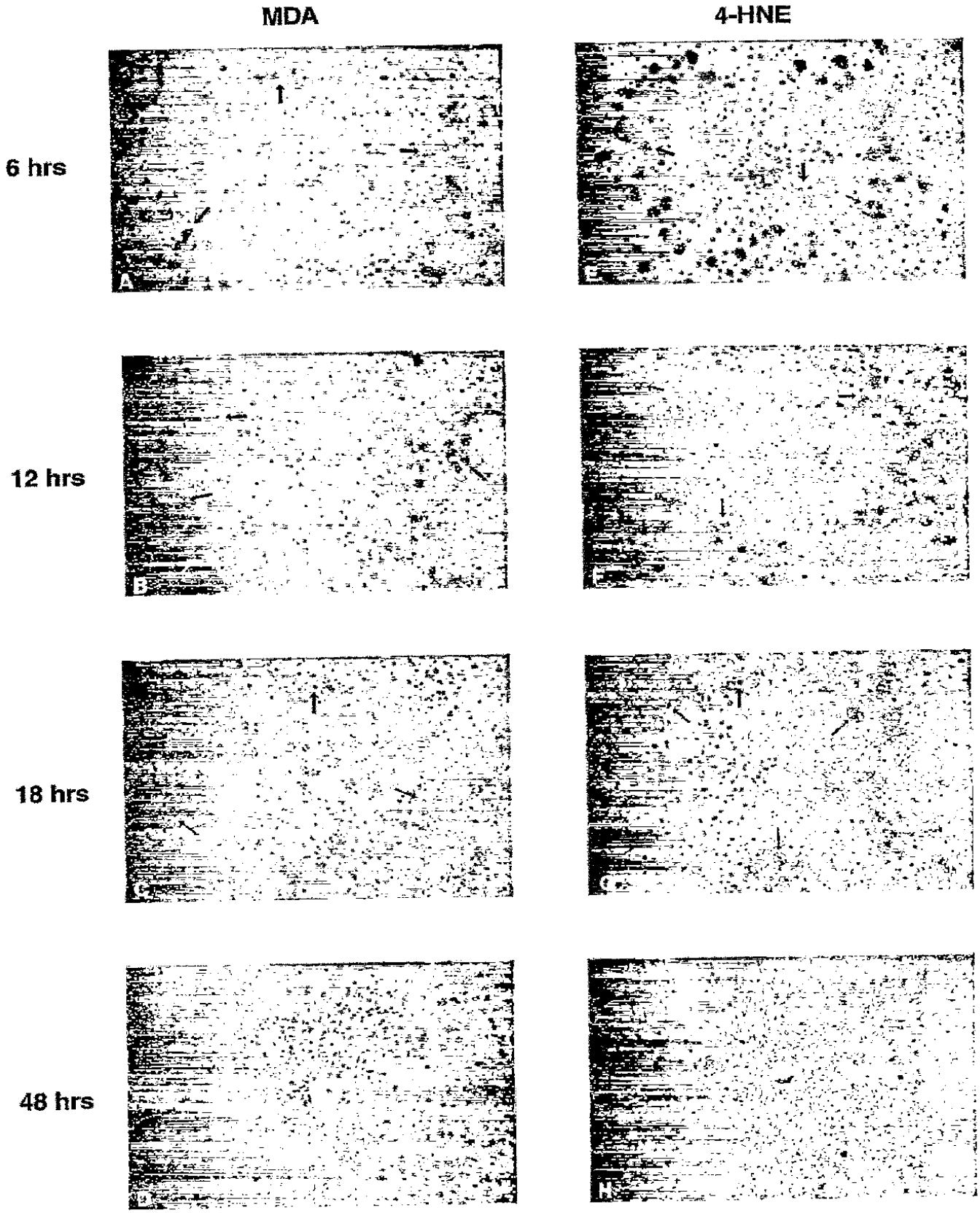


FIG. 3. Accumulation of thiobarbituric acid reactive substances (TBARS) in rat liver after administration of mineral oil alone (solid squares) or exposure to CCl_4 in mineral oil (solid circles). Values represent means ±SEM for n = 3 to 7 determinations at each time point.



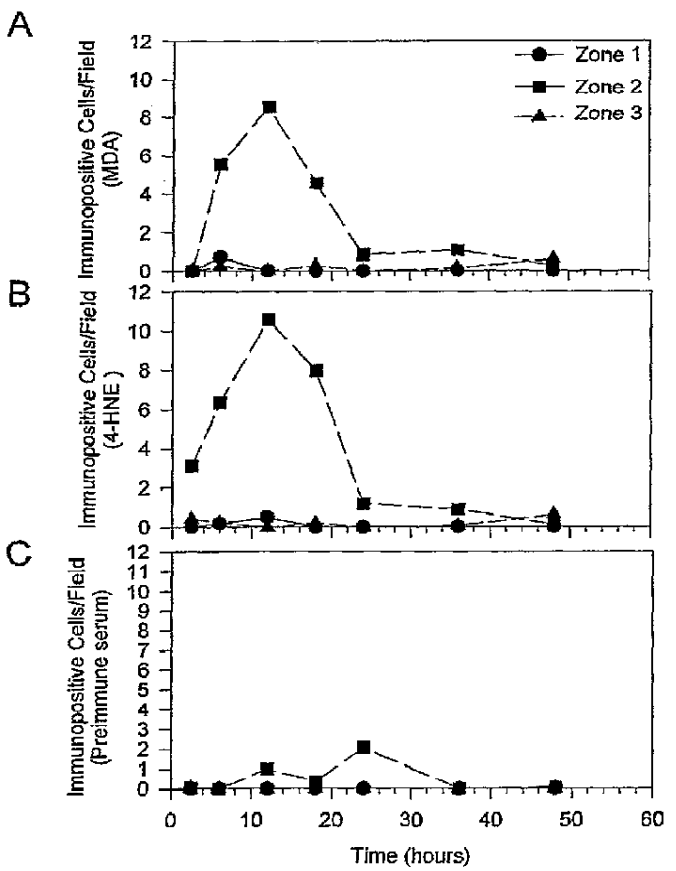


FIG. 5. Quantification of lobular distribution of MDA and 4-HNE immuno-positive cells. (A and B) Profiles of MDA and 4-HNE immuno-positive cells, respectively. (C) Profile of immunopositive cells in liver sections incubated with pre-immune serum, as (−) control. Data represent the average of the number of immuno-positive cells observed in sections prepared and immuno-stained from at least five animals representative of the designated treatment groups.

positive proteins were detected in liver homogenates from CCl_4-treated rats following immuno-precipitation with either the MDA-amine or the 4-HNE-sulfhydryl antiserum. When homogenates from CCl_4-treated rats were immunoprecipitated with anti-MDA-amine serum, immuno-positive proteins of 80, 150, and 205 kDa were apparent. In addition, a number of proteins of greater than 205 kDa were also immuno-positive for MDA adducts. The 80-kDa protein was apparently adducted with MDA as early as 2.5 h after CCl_4 administration, and the 150, 205, and >205 proteins were detected as early as 6.0 h after CCl4 administration. The immuno-reactivity of each of these protein bands increased over the time course to 36 h and diminished thereafter. In Fig. 7, 4-HNE-sulfhydryl immuno-positive proteins of 80, 150, and >205 kDa were detected and increased in intensity after CCl_4, but these proteins were not detected until the 24-h time point. The 205-kDa protein was not apparent in immuno-precipitations using anti-4HNE-sulfhydyl serum. Like MDA-adducted proteins, the 4-HNE-immuno-positive proteins were diminished at 48 h after CCl_4 treatment.

When antiserum to 4-HNE-amine epitopes was used to immuno-precipitate liver homogenates from CCl_4-treated rats, no immuno-positive proteins could be visualized (data not shown). Likewise, no immuno-positive proteins were detected in experiments using preimmune sera in immuno-precipitations with homogenates from CCl_4-treated rats. In a previous study (Hartley *et al.*, 1997) we demonstrated that preincubation of these 4-HNE-sulfhydryl- and MDA-amine-specific antisera with the specific adducts, prior to immuno-precipitation, abolished the ability of these antisera to immuno-precipitate adducted proteins.

DISCUSSION

The data presented here are consistent with a large body of literature documenting the potent and progressive hepatotoxic nature of CCl_4. Following the administration of CCl_4, centrilobular steatosis, an influx of inflammatory cells, and centrilobular necrosis were observed. The incremental histologic changes reported here were associated with increased plasma ALT activities and progressive hepatic lipid peroxidation. These data thus confirm the predictable and temporal relationship between hepatocellular injury and lipid peroxidation in CCl_4-treated rats. Since CCl_4 is a well-known initiator of lipid peroxidation *in vivo*, this agent should mediate formation of 4-HNE and MDA and subsequent alkylation of hepatic proteins with both aldehydic products of lipid peroxidation. Data presented in the present study are consistent with this notion, in that MDA- as well as 4-HNE- protein adducts were documented in liver sections (Fig. 2) and homogenates (Figs. 6 and 7) prepared from CCl_4-treated rats in our study. While the presence of 4-HNE-protein adducts in liver sections of rats treated with CCl_4 has not previously been reported, our detection of MDA-adducted proteins agrees with the results described elsewhere (Bedossa *et al.*, 1994). Also consistent with this previous report is our observation that MDA-adduct for-

FIG. 4. Immuno-histochemical detection of CCl_4-induced formation of hepatic MDA protein adducts. Photomicrographs (magnification 100×) are of representative liver sections obtained from rats given a single intragastric dose of CCl_4 (1.0 ml/kg). Tissue sections were incubated with the MDA (micrographs A–D) or 4-HNE-sulfhydryl antisera (micrographs E–H) and immuno-positive interactions were visualized. A central vein is present in the center of each photomicrograph. Representative tissue sections obtained from individual animals at 6.0 (A and E), 12 (B and F), 18 (C and G), and 48 (D and H) h, respectively, after exposure to CCl_4 are shown. The arrows designate sites of immuno-positive staining localized primarily in the midzonal region.

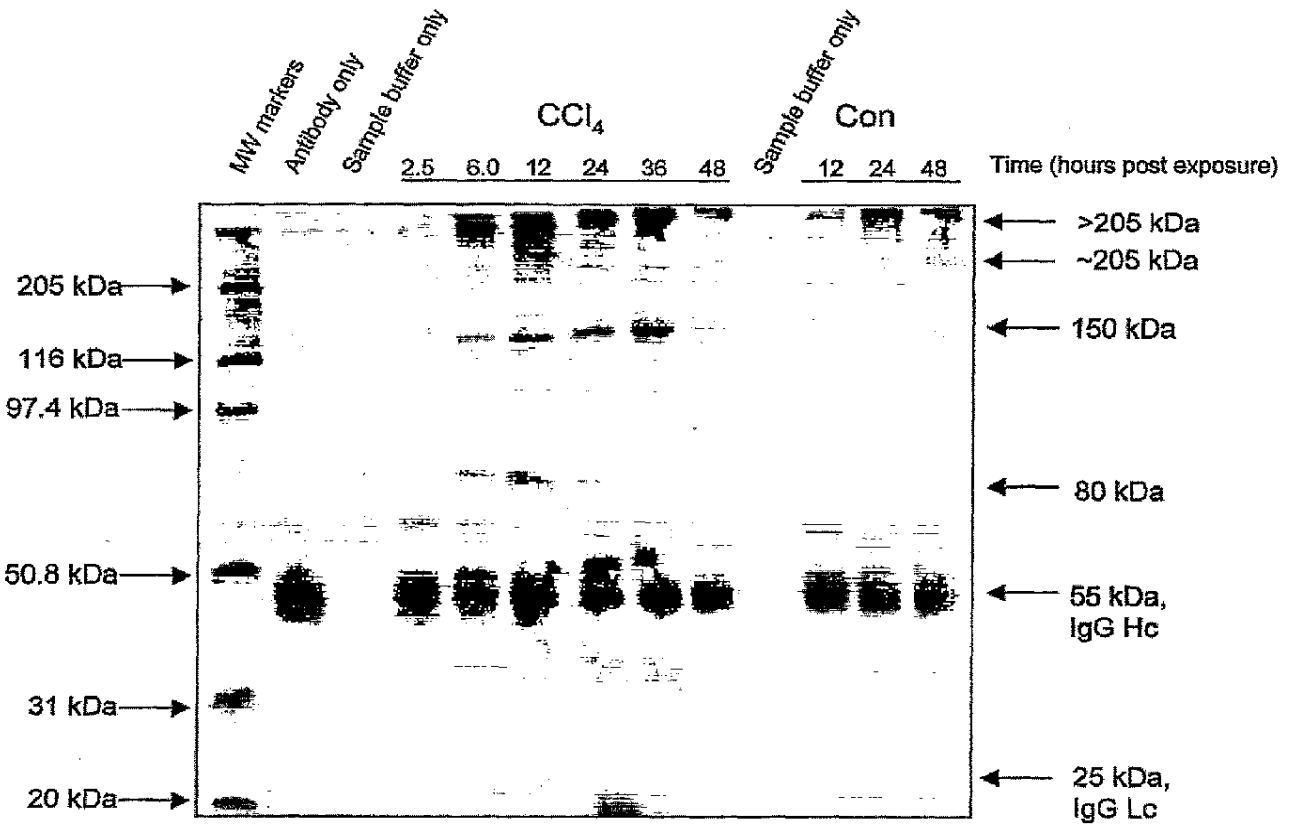


FIG. 6. Detection of proteins alkylated by MDA at various time points after CCl_4 exposure. Representative immuno-precipitation–immuno-blot obtained with the MDA223 antisera (1:500). Lane assignments are as follows: molecular weights of the immuno-positive proteins (right) were determined relative to molecular weight standards in lane 1; lane 2, antibody immuno-precipitated alone; lane 3, sample buffer; lanes 4–9, homogenates immuno-precipitated from animals euthanized 2.5, 6.0, 12, 24, 36, and 48 h after exposure to CCl_4; lane 10, sample buffer only; and lanes 11–13, homogenates prepared from rats 12, 24, or 48 h after administration of mineral oil, The heavy chain (IgGHc) and light chain (IgGLc) of IgG carried through the immuno-precipitation are labeled at 55 and 25 kDa, respectively.

mation in the midzonal region of the hepatic acinus is detectable within 6 h after CCl_4 exposure. In this previous study (Bedossa *et al.*, 1994), formation of MDA-adducts was progressive and by 48 h immuno-positive hepatocytes were continuous from zone 2 to zone 3. These investigators also reported that the density of immuno-positive cells decreased over a 7-day postexposure period. Our results, however, suggest a more rapid onset of hepatotoxicity and lipid aldehyde adduct formation in liver sections prepared from rats euthanized 6–24 h after CCl_4 intoxication. Also, within 48 h, centrilobular necrosis was extensive and aldehyde protein adducts were no longer detectable immunohistochemically (Fig. 2) or by Western analysis of liver homogenates (Figs. 6 and 7). The difference in the formation and elimination rates of hepatic MDA-protein adducts reported here and in the previous report may be due to differences in the strains of rats used, the chemical nature of the carrier vehicle for CCl_4 administration, or immunohistochemical procedures.

In the present study, MDA or 4-HNE-adducted proteins appeared to be intracellular and localized to hepatocytes rather than the less abundant nonparenchymal cells such as Kupffer cells or Ito cells (Fig. 2), which are not necessarily zonal in their localization. Others have reported that, following acute CCl_4 administration, MDA-adducted proteins were localized in hepatocytes (Bedossa *et al.*, 1994). While the presence of 4-HNE-adducted proteins in hepatocytes following CCl_4 administration has not previously been reported, the photomicrographs in Fig. 2 suggest that these adducts colocalize in hepatocytes with MDA-adducted proteins. Published reports describing the cellular localization of 4-HNE- or MDA-adducted proteins in livers of humans or experimental animals experiencing chronic liver injury are variable. For instance, diffuse extracellular and intracellular MDA adducts have been described in livers of rats chronically consuming alcohol (Niemela *et al.*, 1994, 1995). However, these same investigators also observed MDA adducts localized within centrilobular hepatocytes in liver biopsy specimens from human alcoholics or micropigs fed alcohol. More recently, the presence of MDA-

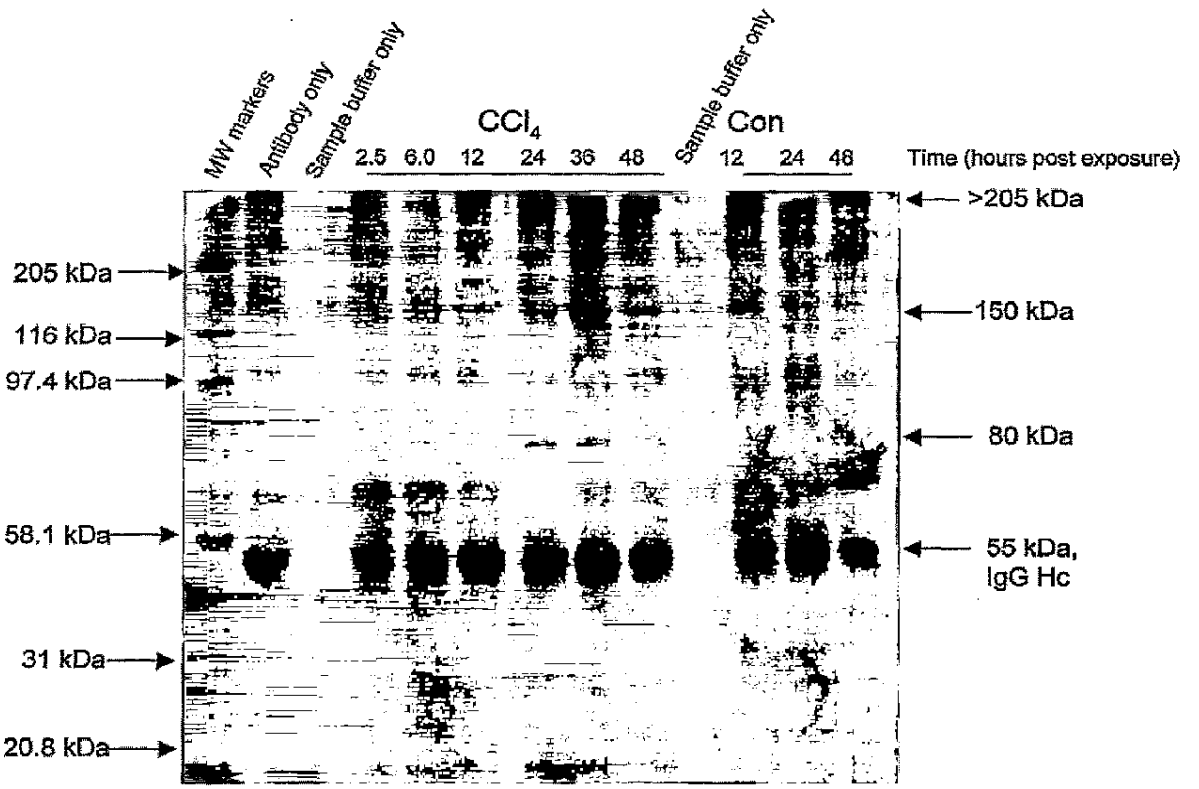


FIG. 7. Detection of proteins alkylated by 4-HNE at various time points after CCl_4 exposure. Representative immuno-precipitation–immuno-blot obtained with the HNESH9402 antisera (1:500). Lane assignments are as follows: molecular weights of the immuno-positive proteins (right) were determined relative to molecular weight standards in lane 1; lane 2, antibody immuno-precipitated alone; lane 3, sample buffer only; lanes 4–9, homogenates immuno-precipitated from animals euthanized 2.5, 6.0, 12, 24, 36, and 48 h after exposure to CCl_4; lane 10, sample buffer only; and lanes 11–13, homogenates prepared from rats 12, 24, or 48 h after administration of mineral oil. The heavy chain of IgG carried through the immuno-precipitation and is labeled at 55 kDa.

and 4-HNE- adducted proteins in biopsy samples obtained from humans having hemochromatosis, Wilson's disease, or alcoholic liver disease were found within the cytoplasm of hepatocytes (Paraids *et al.*, 1997a). Interestingly, liver biopsy samples from patients with chronic hepatitis C revealed the presence of 4-HNE adducts in the cytoplasm of hepatocytes, while MDA adducts were detected in the extracellular matrix localized in areas of periportal or lobular necrosis (Paradis *et al.*, 1997b). Collectively, and in the context of the present study, these reports suggest that the cellular and lobular site of MDA- or 4-HNE-adduct formation is different in acute versus chronic liver injury.

The data presented in Fig. 4 demonstrate that antisera generated to 4-HNE-sulfhydryl epitopes detected immuno-reactive proteins localized within hepatocytes. While the staining of these immuno-reactive proteins was more intense than that observed using MDA antisera, both are localized in the same lobular regions and exhibit the same time course of appearance as 4-HNE-adducts. It is interesting to note that Bedossa *et al.* (1994) did not detect 4-HNE-protein adducts in liver sections from CCl_4-treated rats. A potential explanation for this apparent contradiction is likely related to the fact that these investigators used antibodies directed to 4-HNE-amine epitopes while we employed antibodies prepared against 4-HNE-sulfhydryl epitopes. In support of this proposition is the observation in the present study that antisera to 4-HNE-amine epitopes did not detect immuno-reactive epitopes in these tissue sections. In addition, 4-HNE-sulfhydryl antiserum did not recognize 4-HNE-amine epitopes (i.e., bovine serum alkylated with 4-HNE) in ELISA analyses, and our 4-HNE-amine antiserum was not effective in immuno-precipitating 4-HNE-adducted proteins in liver homogenates from CCl_4-treated rats (data not shown). As noted in a comprehensive review, adduct formation of 4-HNE with protein sulfhydryl groups occurs very readily at a pH of 7.4 and low concentrations of reactants (Esterbauer *et al.*, 1991). The functional groups of histidine, lysine, and glycine react with 4-HNE. However, these reactions proceed most rapidly under *in vitro* conditions (i.e., pH 8.8 and 10 mM reactants), which are not physiologically relevant. These results suggest that 4-HNE produced *in vivo* may preferentially alkylate sulfhydryl groups of hepatocellular proteins.

The data presented here also demonstrate that, following

CCl_4 administration to rats, MDA- and 4-HNE-adducted proteins colocalize in zone 2 of the hepatic lobule. This pattern of MDA- and 4-HNE-adduct formation and colocalization was apparent within 6 h following treatment, appeared maximal at 24 and at 36 h, and resided primarily in zone 2 or zone 3 hepatocytes displaying altered morphology. In this context, the time course of appearance and lobular localization of the MDA-adducted proteins is consistent with that reported elsewhere (Bedossa *et al.*, 1994). Additionally, the data presented here are the first to document that 4-HNE-adducted proteins are formed during the same time course and occur in the same lobular distribution as MDA-adducted proteins. The unique cellular or biochemical characteristics that predispose these specific hepatocytes to prooxidant injury mediated by CCl_4 remains to be determined. One likely explanation is that these hepatocytes are exposed to increased oxygen tension and/or can be distinguished by a profile of enzymes that enhance the prooxidative cellular injury of CCl_4. It is also noteworthy that, while the aldehyde-protein adducts were localized to zone 2, necrosis was most prominent in zone 3. This observation suggests a potential dissociation between adduct formation and the zonal necrosis characteristic of CCl_4 intoxication. Thus a direct, mechanistic link between adduct formation and hepatocellular necrosis resulting from CCl_4 remains to be established.

A significant effect was associated with mineral oil and corn oil exposure where formation of aldehyde protein adducts is apparent in tissue sections and in immuno-blots. The present findings are likely related to the oxidation of these oils and formation of oil-derived aldehydic products of lipid peroxidation. Indeed, significant amounts of aldehydic products of lipid peroxidation have been detected in various oils (Lang *et al.*, 1985). Also, 4-HNE from oxidized anilide oils was postulated to regulate the onset of fibrogenesis observed in Toxic Oil Syndrome (Hernandez-Monz *et al.*, 1994).

The results presented in the current investigation are novel in that they further extend previous reports of hepatic aldehyde-protein adducts in liver tissue sections by using procedures to detect specific MDA- and 4-HNE immuno-reactive hepatic proteins in liver homogenates. As noted in Figs. 6 and 7, specific immuno-reactive proteins of 80, 150, and ≥205kDa were consistently detected in liver homogenates prepared from rats exposed to CCl_4 and, to a lesser degree, in rats that received mineral oil. In samples from both mineral oil- and CCl_4-treated rats, the formation of adducted proteins was time dependent. In general, in samples from CCl_4-treated rats, MDA and 4-HNE immuno-reactive proteins of molecular weights of 80, 150, and ≥205 kDa displayed increasing optical densities with time up to 36 h and decreased thereafter. It is intriguing that, in homogenates, lipid aldehydes are evident 36 and 48 h after exposure to CCl_4, however, the presence of detectable adducts in tissue sections is minimal. Whether these adducts are cleared from the liver as a result of cellular necrosis or packaged in subcellular organelles and, as such, are undetectable by our immuno-histochemical procedures is not known and will be elucidated in future studies.

In the present study, detection of 80- and 150-kDa aldehyde-adducted proteins in liver homogenates of CCl_4-treated rats is consistent with the array of MDA- or 4-HNE-adducted proteins that were detected in isolated rat hepatocytes during the time course of CCl_4-stimulated lipid peroxidation (Hartley *et al.*, 1997). In addition, the appearance of these same proteins in control (mineral oil) samples and in control hepatocytes are consistent findings, which suggest that these proteins are very sensitive and specific markers of oxidative stress.

To date, there are no published reports concerning the identity of hepatic proteins adducted by MDA or 4-HNE. However, in the case of certain forms of experimental nephritis thought to be mediated by reactive oxygen species and lipid peroxidation, a major glomerular basement membrane 220-kDa protein adducted with MDA has been identified as type IV collagen (Neale *et al.*, 1994). The identification and further characterization of the proteins described in the present study is currently under way and will be essential to establish their roles in CCl_4-induced liver damage.

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